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Visualization of Peptide Secretory Vesicles in Living Nerve Cells

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Abstract

Analysis of real-time movements of peptidergic vesicles in live neurons provides insight into molecular mechanism(s) supporting the activity-dependent secretion of neurotrophins and neuropeptides. We examined the effect of overexpression of exogenous peptides comprising of the cytoplasmic tail sequence of vesicular carboxypeptidase E (CPE), proposed to be involved in the mechanism of trafficking of peptidergic secretory vesicles, in live hippocampal neurons. E16 rat hippocampal neurons were transfected with the peptidergic vesicle markers, CPE C-terminally tagged with red or green fluorescent protein, or brain-derived neurotrophic factor (BDNF) tagged with green fluorescent protein, and grown on dishes specialized for real-time live cell visualization. Movements of peptidergic vesicles were imaged in a temperature-controlled chamber on a confocal inverted microscope and analyzed with respect to their velocity, displacement distance, and processivity.

Keywords

Carboxypeptidase E; Brain-derived neurotrophic factor; Hippocampal neurons; Peptidergic secretory vesicles; Vesicle trafficking

1. Introduction

Post-Golgi anterograde transport of peptidergic vesicles to the secretion sites at the nerve terminals is a prerequisite for the activity-dependent secretion of neurotrophins and neuropeptides for neurotransmission or neuromodulation in various areas of the brain, such as the hippocampus (1–3). Conversely, retrograde transport of the peptidergic vesicles is also important for delivery of neurotrophin-based survival signals to the cell body, as well as for clearance and recycling of membrane proteins at the synaptic terminals (3). Our previous studies have shown that peptidergic vesicles in neuroendocrine cells and hippocampal neurons contain a transmembrane form of the prohormone processing enzyme, carboxypeptidase E (CPE), with a cytoplasmic tail that interacts with microtubule-based motors to effect vesicle transport (4). The methods for examining the molecular mechanism(s) by which neurons mediate peptidergic vesicle transport, involve conducting live neuron imaging using rat hippocampal neurons to visualize real-time movement of peptidergic vesicles, in the absence, and in the presence of peptides comprising of the CPE cytoplasmic tail amino-acid sequence that is expected to interfere with transport of vesicles containing both CPE and brain-derived neurotrophic factor (BDNF). In this chapter, we

describe in detail the methods used for the live neuron imaging (4). Briefly, E18 rat hippocampal neurons were transfected with the peptidergic vesicle markers, CPE tagged with either GFP (CPE-GFP), red fluorescent protein (CPE-RFP), or BDNF tagged with green fluorescent protein (BDNF-GFP). Along with the peptidergic vesicle markers, we transfected the cytoplasmic tail of CPE N-terminally tagged with either GFP or RFP (GFP-CPE_{C25} or RFP-CPE_{C10}), which was expected to affect real-time trafficking of CPE/BDNF vesicles (4). Transfected live neurons were seeded on the delta TPG dish specialized for live cell imaging and imaged for ~6.7 min using confocal microscopy. The neuron images were analyzed with respect to the velocity, and the total distance and steps each peptidergic vesicle traveled in live neurons. The methodology described in this chapter may be used for following vesicle transport in any neuron transfected with any fluorescent-tagged vesicle protein of interest.

2. Materials

2.1. Neuron Transfection and Culture

2.1.1. Hippocampal Neuron Culture

1. E18 rat primary hippocampal neurons (Gene Therapy Systems, San Diego, CA) (see Note ¹). Cultures should be ordered 1 week before transfection.
2. 0.25 M Borate solution (Sigma Chemicals, St. Louis, MO): Dilute in deionized water to make 0.1 M Borate buffer.
3. 0.01% Poly-L-lysine (Sigma Chemicals): Dilute in 0.1 M Borate buffer to make 0.001% poly-L-lysine prior to use (see Note ²).
4. 0.17-mm black delta TPG dishes (Bioprotech, Inc., Butler, PA) (see Note ³).
5. HEPES-based saline buffer (HBS).
6. Phenol red-plus/free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (see Note ⁴).
7. Neurobasal/B27 medium (GIBCO-BRL, Life Technologies, Bethesda, MD) (see Note ⁵).

2.1.2. DNA Constructs

1. A 5'-XhoI-PstI-3' digest of PCR product of CPE tail fragments (CPE_{C25} or CPE_{C10}) amplified from full-length CPE cDNA is subcloned into pEGFP-C1 or pDsRed2 (BD Bioscience, San Diego, CA) to generate GFP-CPE_{C25} or RFP-CPE_{C10}.

¹The company dissects hippocampal neurons from rat on Wednesday and delivers the neurons on Thursday.

²Borate buffer is recommended by many different neuroscience research laboratories for coating the plate/coverslip for neurons. Neurons are easily detached from the dish coated with 0.001% poly-L-lysine in PBS or HBS during procedure.

³Black delta dish is recommended rather than transparent dish because the fluorescence in live cells is more easily bleached by any light source.

⁴Phenol red-free DMEM is required for acquisition of red fluorescence to avoid interference by the red color of the phenol red.

⁵Neurobasal medium usually comes with delivery of rat hippocampal neurons so that it is not necessary to order it separately.

2. Full-length CPE cDNA is excised from pEGFP-N1 and inserted into pDsRed-Express-N1 (BD Bioscience Clontech Co.) to generate CPE-RFP.
3. BDNF-GFP is a gift from Dr. Bai Lu (Glaxo Smith Kline, China) (see Note ⁶).

2.1.3. Electroporation

1. Rat Neuron Nucleofector[®] kit (Amaxa Inc., Gaithersburg, MD) (see Note ⁷).
2. Electroporation device (Amaxa Nucleofector, Amaxa Inc.).
3. 2-mm-gap electroporatable cuvettes (Amaxa Inc.).
4. 2-mL plastic spoid pipets (Amaxa Inc.).

2.2. Live Neuron Imaging and Analysis

1. Zeiss Axiovert 200 M inverted microscope (Carl Zeiss, Thornwood, NY) equipped with 63× Zeiss planapochromat oil, 1.4 NA, DIC and 100× Zeiss alpha plan fluor oil, 1.45 NA, DIC objectives. The microscope is also equipped with a closed chamber that is connected to a hot air blower controlled by a temperature sensor.
2. “LSM 510 Meta” software version 3.5 (Carl Zeiss).
3. “Metamorph” software (Molecular Devices, Downingtown, PA).

3. Methods

Compared to real-time imaging of fibroblasts and epithelial cells that are easy to transfect and manipulate, live cell imaging of neurons should be executed in a timely and punctual manner. Moreover, intracellular events including trafficking of peptidergic secretory vesicles in neurons are very sensitive to the environment, such as changes in temperature and air composition; any delay in preparation and execution should be avoided during the whole procedure. To obtain good resolution for individual vesicle movement, the levels of expression of fluorescent protein should be optimized by adjusting the amount of DNA used for transfection based on the strength of the promoter for fluorescent protein expression. It is also important to allow enough time after transfection of the neurons for neurite outgrowth, while posttransfection time should not be too long to over-saturate the capacity of the neurons with exogenous proteins. To achieve vesicle movements in the environment closest to physiological condition, only one live neuron should be imaged from one dish that is just removed from the incubator to a pre-warmed microscope.

During image analysis of real-time movies, it is hard to track a complete set of movements of single vesicle using an automated program such as “Single Particle Tracking” of “Metamorph” software because the tracking program loses its target easily when other vesicles cross over the region of interest on a target vesicle. The way to track a target vesicle

⁶Red fluorescence yields a stronger signal than green fluorescence, but offers less resolution in live cells. This may be due to a stronger innate intensity of red fluorescence than green fluorescence.

⁷Mouse Neuron Nucleofector kit[®] also works for transfection of rat neurons.

from the beginning to the end of the movement is to follow the movement of a single vesicle by marking each movement using the method described by King et al. (5).

3.1. Neuron Transfection and Culture

1. One day before neuron delivery, coat the bottom of the delta TPG dish with 1 mL of 0.001% poly-L-lysine in 0.1 M borate buffer overnight (see Note ⁸).
2. Remove poly-L-lysine from the dish.
3. Wash the dish surface with 1 mL of HBS buffer twice.
4. Add 1 mL of DMEM plus 10% FBS into the dish and incubate in 37°C + 5% CO₂ incubator before use.
5. Upon neuron delivery, transfer the neurobasal medium containing E18 rat hippocampal neurons (2×10^6 neurons) into a 15-mL conical tube (see Note ⁹).
6. Completely dissociate neurons by gentle pipetting (see Note ¹⁰).
7. Spin down dissociated neurons in a bench top centrifuge for 10 min at $700 \times g$. Carefully remove the supernatant over neuron pellets by gentle pipetting (see Note ¹¹).
8. Resuspend pelleted neurons in 200 μ L of rat neuron nucleofector solution for electroporation and split into two 1.5-mL tubes (100 μ L per tube). Each tube will contain 1×10^6 neurons.
9. 10 μ g/10 μ L of control vector DNA (5 μ g/5 μ L) (pEGFP or pDsRed alone) or peptide inhibitor-encoding DNA (GFP-CPE_{C25} or RFP-CPE_{C10}) along with DNA (5 μ g/5 μ L) for vesicle marker (CPE-RFP or BDNF-GFP) is added into 100 μ L of neuron suspension.
10. Transfer 110 μ L of the mixture of DNA and neurons to a 2-mm-gap electroporatable cuvette (see Note ¹²).
11. Perform electroporation of neurons using the program C-13 in the Amaxa nucleofector device (see Note ¹³).
12. Immediately after electroporation, slowly and gently drop neurons onto 1 mL of pre-warmed 10% FBS + DMEM medium in a delta TPG dish using a 2-mL plastic spoid pipet (see Note ¹⁴).

⁸The capacity of delta TPG dish is 1 mL.

⁹Delivered neurons can be kept at 4°C for several hours, but longer storage decreases the survival rate of neurons after electroporation.

¹⁰While dissociation solution containing papain is recommended for neuron dissociation by the company, it should not be used for neuron dissociation. Use of the solution results in death of more than 90% of the neurons after electroporation. It may be due to influx of proteases in the dissociation buffer into neurons upon electroporation.

¹¹The size of neuron pellet is very small so that the pellet is easily sucked up by rough pipetting.

¹²The electroporation cuvette with DNA and neuron mixture should be gently tapped to cause the mixture to settle to the bottom of the cuvette.

¹³Other electroporation programs do not yield better transfection efficiency than C-13.

¹⁴White debris (dead neurons) floating on the top of electroporated neurons should be removed before transferring live neurons into a delta T dish.

13. Incubate neurons within the TPG dish in DMEM plus 10% FBS for 2 days. This incubation step is for recovery of neurons from the electric shock of electroporation (see Note 15).
14. Replace DMEM with neurobasal/B27 medium and incubate neurons for an additional 2 days. Transfection efficiency of <10% is expected.

3.2. Live Neuron Imaging and Analysis

3.2.1. Imaging

1. Pre-warm the closed chamber on the Zeiss Axiovert 200 M inverted microscope to 37°C 30 min prior to live neuron imaging.
2. After temperature inside the chamber reaches 37°C, place a delta TPG dish containing transfected neurons on the stage of the microscope (see Notes 16 and 17). Figure 1 shows the best example of a neuron that has both an optimum level of fluorescent protein-containing peptidergic vesicles and well-developed neurites for live neuron imaging.
3. Image neurons expressing CPE-RFP and GFP/GFP-CPE_{C25} or BDNF-GFP and RFP/RFP-CPE_{C10} using “LSM 510 Meta” software at 1.97-s exposure/shot for 200 shots. Supplemental movie S1 shows bi-directional movements of CPE-RFP containing peptidergic vesicles along the neurites in live hippocampal neurons expressing GFP alone.
4. After taking 200 images of vesicle movements for 394 s, turn on the hot air blower connected to microscope chamber for the next live neuron imaging.

3.2.2. Analysis

1. Import a set of 200 images into “Metamorph” software.
2. Use Pixels in the scale bar on the image generated in “LSM 510 Meta” software to calculate $\mu\text{m}/\text{pixel}$ using “Metamorph” software (4, 6).
3. Determine the displacement of CPE-RFP/BDNF-GFP vesicle on the trajectory route on the basis of x - and y -coordinates. The total number of pixels per each displacement is converted to micrometer.
4. Calculate the velocity of each vesicle movement by dividing the length (μm) of each displacement with an interval of 1.97 s.
5. Calculate the average speed of movements of each vesicle by averaging all the instant velocities shown by a single vesicle.
6. Calculate the total distance from the summation of all the displacements each vesicle moved. Figure 2 shows the summary of average speeds and total distances

¹⁵The incubation in FBS-DMEM for longer or shorter than 2 days does not enhance the transfection efficiency.

¹⁶The time for transfer of the dish containing live neurons from incubator to microscope and finding a neuron that expresses both GFP and RFP proteins at optimum levels, and has well-developed neurites should be minimized to less than 4 min.

¹⁷Before starting acquisition of real-time images, the hot air blower should be turned off to prevent effect of air current on the oil between the top of the objective and the bottom of the dish.

of movements of CPE-RFP-containing peptidergic vesicles in hippocampal neurons expressing either GFP or GFP-CPE_{C25}. Peptidergic vesicles containing CPE-RFP move at different speeds in the presence of GFP or GFP-CPE_{C25}.

7. Count the total step (interval) numbers from all the displacements of each CPE-RFP-containing peptidergic vesicle in the presence of GFP or GFP-CPE_{C25}. Figure 3 shows the summary of the total step (interval) numbers counted from all the displacements of CPE-RFP-containing peptidergic vesicles in the presence of GFP or GFP-CPE_{C25}.
8. Sort separately among each other vesicles moving in either anterograde or retrograde direction with respect to the total number of time intervals (1.97 s) each vesicle moved.
9. Calculate the average speed, total distance, and total step numbers of movements of BDNF-GFP-containing vesicles in RFP or RFP-CPE_{C10}-expressing neurons in the same way as above.
10. Carry out statistical analysis using the Student *t*-test (two tailed, non-paired) and report the mean values $\pm$ SEM for the average of total distances, total steps, and mean velocities that the vesicles move.

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Fig. 1.

Confocal image of a hippocampal neuron expressing CPE-RFP in peptidergic vesicles. E18 rat hippocampal neurons were transfected with CPE-RFP via electroporation, seeded on a poly-L-lysine-coated coverslip, and incubated in DMEM plus 10% FBS for 2 days for recovery. Then, the DMEM was replaced with neurobasal medium and incubated for 2 more days for neuron differentiation. Neurons on the coverslip were fixed in 3.5% paraformaldehyde and mounted on a slide for confocal microscopy (reproduced from ref. 5 with permission from Elsevier Science). Scale bar = 5 μ m.

Fig. 2.

Graphs showing analyses of live in vivo movement of CPE-RFP vesicles in hippocampal neurons. The velocity of vesicle movement was calculated from each displacement per 1.97 s, while the total distance was the summation of all the displacements each vesicle moved. Vesicles moving in either anterograde or retrograde direction with respect to the total number of time intervals (1.97 s) each vesicle moved were classified separately (reproduced from ref. 5 with permission from Elsevier Science).

Fig. 3.

Bar graphs showing the number of vesicles moved in each time interval ($1.97 \text{ s} \times \text{number of steps}$). Vesicles moving in either anterograde or retrograde direction with respect to the total number of time intervals (1.97 s) each vesicle moved were classified separately (reproduced from ref. 5 with permission from Elsevier Science).